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Genes Similar to the *Vibrio parahaemolyticus* Virulence-Related Genes *tdh*, *tlh*, and *vscC2* Occur in Other *Vibrionaceae* Species Isolated from a Pristine Estuary

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Detection of the human pathogen *Vibrio parahaemolyticus* often relies on molecular biological analysis of species-specific virulence factor genes. These genes have been employed in determinations of *V. parahaemolyticus* population numbers and the prevalence of pathogenic *V. parahaemolyticus* strains. Strains of the *Vibrionaceae* species *Photobacterium damselae*, *Vibrio diabolicus*, *Vibrio harveyi*, and *Vibrio natriegens*, as well as strains similar to *Vibrio tubiashii*, were isolated from a pristine salt marsh estuary. These strains were examined for the *V. parahaemolyticus* hemolysin genes *tdh*, *trh*, and *tlh* and for the *V. parahaemolyticus* type III secretion system 2α gene *vscC2* using established PCR primers and protocols. Virulence-related genes occurred at high frequencies in non-*V. parahaemolyticus Vibrionaceae* species. *V. diabolicus* was of particular interest, as several strains were recovered, and the large majority (>83%) contained virulence-related genes. It is clear that detection of these genes does not ensure correct identification of virulent *V. parahaemolyticus*. Further, the occurrence of *V. parahaemolyticus*-like virulence factors in other vibrios potentially complicates tracking of outbreaks of *V. parahaemolyticus* infections.

Vibrio parahaemolyticus is the leading cause of seafood-associated gastroenteritis in the United States and the world (1). The U.S. Food and Drug Administration (1) estimates that 4,500 reported cases of *V. parahaemolyticus* gastroenteritis occur every year, and outbreaks of *V. parahaemolyticus* infections are increasing in frequency and expanding in geographic range (2, 3). This organism is ubiquitous in nearshore marine waters, and cell numbers are typically highest in surficial sediments (4) and in turbid waters bearing high loads of resuspended sediment (5, 6). Filter-feeding bivalve mollusks, such as oysters and mussels, can concentrate *V. parahaemolyticus* and other pathogenic vibrios (for examples, see references 7 and 8), resulting in levels in the mollusks capable of producing infection in a person that ingests them (9). Virulent *V. parahaemolyticus* strains are clearly a concern for seafood safety, and their detection is important anywhere that elevated levels of this organism are found.

Detection of *V. parahaemolyticus* in shellfish and environmental samples is typically based on molecular biological analysis of specific genes, particularly genes exclusive to this species and those strongly correlated with pathogenicity. The gene encoding the thermolabile hemolysin (TLH), designated *tlh*, encodes a phospholipase A2 (10). While its contribution to *V. parahaemolyticus* pathogenicity is unknown, expression of this gene is upregulated under conditions mimicking the human intestine (11, 12). *tlh* is considered to be a species-specific marker for *V. parahaemolyticus* (13, 14) and is frequently employed to identify this species (1, 13, 15–18). Genes encoding the thermostable direct hemolysin (TDH) and the homologous thermostable direct hemolysin-related hemolysin (TRH), *tdh* and *trh*, respectively, have been implicated in *V. parahaemolyticus* virulence (19, 20, 21). TDH and TRH are tetrameric proteins that act as porins and facilitate efflux of divalent cations and other solutes from and influx of water molecules into intestinal cells (11, 22, 23). Occurrence of *tdh* is correlated with the Kanagawa phenomenon (KP), a beta-hemolytic reaction on saline blood agar (Wagatsuma agar) (19). Numerous studies indicated that *tdh* and *trh* are found almost exclusively in clinical strains isolated from patients suffering from *V. parahaemolyticus* gastroenteritis (19, 21). Only about 1 to 2% of screened *V. parahaemolyticus* strains not derived from infected humans (i.e., environmental strains) were reported to carry these genes (24). These results were due, at least in part, to inadequate methodology, and recent studies have shown that high levels of environmental *V. parahaemolyticus*, 52% of strains from an area supporting intensive shrimp aquaculture (25) and 48% of strains from water and sediment in a pristine estuarine ecosystem (26), carry these genes. In addition, *V. parahaemolyticus* strains can carry two type three secretion systems, T3SS1 on chromosome 1 and T3SS2 on chromosome 2 (27). T3SS1 has been found in all tested strains of *V. parahaemolyticus*, while T3SS2 has been reported in virulent strains (27, 28). T3SSs are composed of 20 to 30 proteins and are responsible for translocating effector proteins directly into host cell cytoplasm. There are more than 100 described effector proteins having effects ranging from autophagy to cytotoxicity (29, 30). Additionally, there are two distinct types of T3SS2 (31). T3SS2α has been found in strains that carry *tdh*, while T3SS2β is correlated with *trh* (31, 32). Pathogenesis of *V. parahaemolyticus* does not appear to rely solely on a given virulence function; rather, virulence is complex and different strains may employ somewhat different strategies.

The utility of any of these genes as a molecular marker for *V. parahaemolyticus* or for evaluation of the potential pathogenicity of *V. parahaemolyticus* strains relies upon their specificity for *V.*

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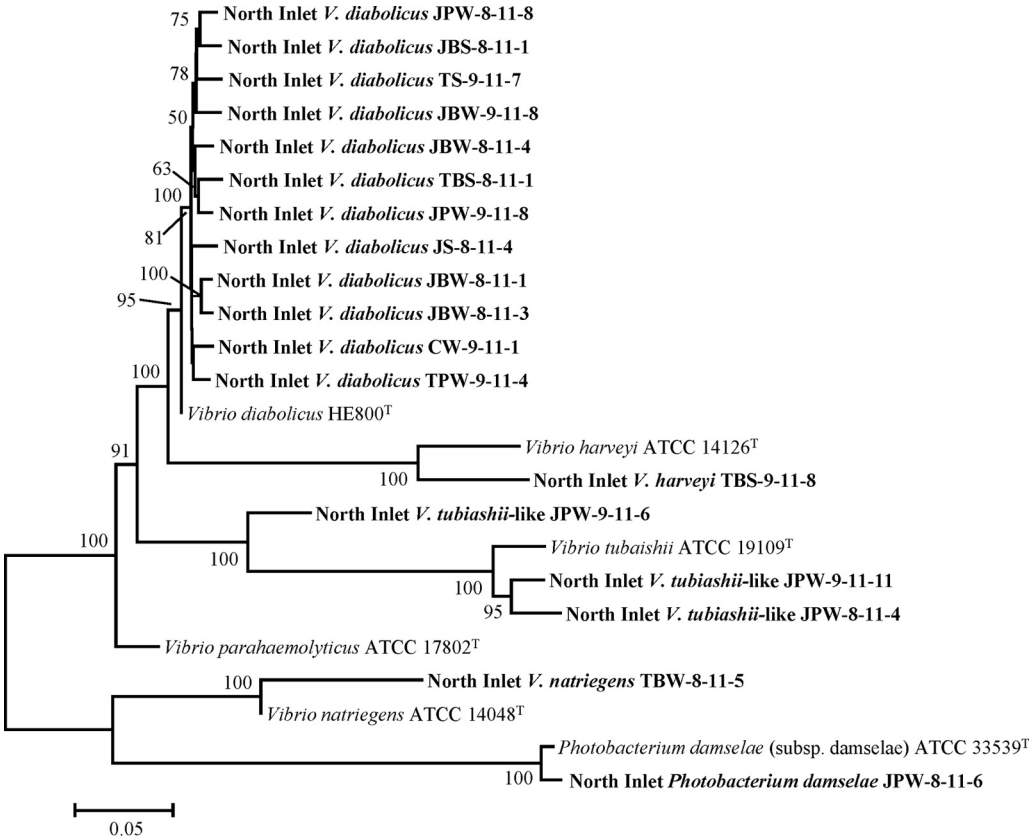

FIG 1 Neighbor-joining phylogeny (Kimura 2-parameter model) of concatenated 16S rRNA gene, *recA*, *rpoA*, and *gyrB* sequences from environmental *Vibrionaceae* strains isolated from the North Inlet estuary. Bootstrap values represent 1,000 replications, and values less than 50 are not shown. Reference sequences were acquired from the NCBI GenBank.

parahaemolyticus. Sporadic reports of *V. parahaemolyticus*-like virulence genes in other species have appeared in the literature (33–39), but little is known regarding the distributions of these genes among *Vibrionaceae*. In particular, the occurrence of these genes in environmental strains has received very little attention. We screened a collection of *Vibrionaceae* strains isolated from a pristine estuary for *tlh*, *tdh*, and *trh* and for a gene encoding a T3SS2α outer membrane protein, *vscC2* (VPA1339), in order to evaluate the specificity of these genes for *V. parahaemolyticus*. Our results show that *tlh*, *tdh*, and *vscC2* are not found exclusively in *V. parahaemolyticus*.

MATERIALS AND METHODS

Sample site and strain isolation. Strains were isolated from the pristine North Inlet estuary near Georgetown, SC. The North Inlet-Winyah Bay National Estuarine Research Reserve protects the third largest watershed on the east coast of the United States, and 90% of the 18,916 acres are in their natural state. The keystone macrophyte *Spartina alterniflora* dominates the intertidal marsh, except at lower-salinity, higher elevations, where the subdominant *Juncus roemerianus* thrives. Fiddler crabs of the genus *Uca* are the biomass dominant fauna within the marsh. Their burrows are found throughout the intertidal marsh and have been shown to contain high levels of *V. parahaemolyticus* (40).

Sampling trips were made in August 2011 and again in September 2011 to coincide with periods of elevated *Vibrio* numbers (4, 40). Samples were collected at low tide from bulk sediment, *Uca* burrow lining sediment, *Uca* burrow water, sediment pore water, and creek water as described previously (4, 40). All samples were diluted with phosphate-buffered saline (400 mM NaCl, 1.75 mM $NaPO_4$, pH 7.4) and plated on thiosulfate citrate bile salts sucrose (TCBS) agar (BD, NJ) by following the FDA protocol (41) without the use of enrichment media. The TCBS plates were then incubated at 37°C for 48 h. Well-isolated colonies were streaked onto fresh TCBS plates for further characterization. Green colonies (typical appearance of *V. parahaemolyticus*) were collected and routinely cultivated on saline Luria agar (SLA; per liter, 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto agar).

Strain designations indicate source (S, sediment; BS, burrow lining sediment; BW, burrow water; PW, porewater; and CW, creek water), the month and year it was isolated (8 to 11 for August 2011 and 9 to 11 for September 2011), and the isolate number. For example, JS-8-11-4 refers to isolate number 4 from *Juncus* zone sediment (JS) collected in August 2011 (8–11).

Physiological characterization. Strains were tested for β-galactosidase activity, sucrose fermentation, and the ability to grow at 42°C (26). β-Galactosidase activity was determined (Oxoid, Hampshire, England) using cultures grown in 1 ml saline Luria broth (SLB) supplemented with 0.1% lactose and incubated at 37°C with shaking. Sucrose fermentation was determined in 1-ml broth cultures (per liter, 10 g casein digest peptone, 0.018 g phenol red, 5 g sucrose, 27 g NaCl) grown at 37°C. The ability of each strain to grow at 42°C was determined on SLA plates. Green colonies on TCBS agar having the ability to grow at 42°C, lacking β-galactosidase activity, and lacking sucrose fermentation to acid end products in broth were presumed to be *V. parahaemolyticus* (42).

Wagatsuma agar. Preparation of Wagatsuma agar followed the FDA bacteriological analytical manual and employed rabbit erythrocytes (41). After curing overnight, each plate was inoculated with one *Vibrionaceae* strain. Inoculated plates were incubated for up to 48 h at 37°C, and isolates

TABLE 1 Occurrence of genes and phenotypic characteristics considered diagnostic for virulence in *V. parahaemolyticus* and also found in non-*V. parahaemolyticus* environmental *Vibrionaceae* strains

Strain designation	Species	Presence of[a]:				KP	Swarming motility	Colony appearance on TCBS agar	β-Galactosidase activity	Sucrose fermentation	Growth at 42°C
		tlh	*tdh*	*trh*	*vscC2*						
JBW-8-11-1	*V. diabolicus*	*	+	−	+	+	+	Green	−	−	+
JPW-9-11-11	*V. tubiashii*-like	+	*	−	+	+	−	Green	−	+	+
JPW-9-11-8	*V. diabolicus*	+	−	−	−	−	+	Green	−	−	+
JPW-8-11-8	*V. diabolicus*	+	−	−	+	−	+	Green	−	−	+
JPW-8-11-6	*P. damselae*	+	−	−	+	−	−	Green	−	−	+
JPW-9-11-6	*V. tubiashii*-like	*	−	−	−	+	−	Green	−	+	+
JBW-8-11-3	*V. diabolicus*	−	+	−	+	−	+	Green	−	−	+
CW-9-11-1	*V. diabolicus*	−	+	−	+	−	+	Green	−	−	+
JS-8-11-4	*V. diabolicus*	−	+	−	−	+	−	Green	−	−	+
TPW-9-11-4	*V. diabolicus*	−	*	−	−	−	+	Green	−	+	+
TBW-8-11-5	*V. natriegens*	−	−	−	+	−	−	Green	−	−	+
JBW-8-11-4	*V. diabolicus*	−	−	−	+	−	+	Green	−	−	+
JPW-8-11-4	*V. tubiashii*-like	−	−	−	+	−	−	Green	−	−	+
JBW-9-11-8	*V. diabolicus*	−	−	−	+	+	+	Green	−	−	+
TS-9-11-7	*V. diabolicus*	−	−	−	+	−	+	Green	−	+	+
TBS-9-11-8	*V. harveyi*	−	−	−	+	+	−	Green	−	+	+
JBS-8-11-1	*V. diabolicus*	−	−	−	−	+	−	Green	−	−	+
TBS-8-11-1	*V. diabolicus*	−	−	−	−	−	+	Green	−	−	+

[a] An asterisk designates a faint amplicon detected but no sequence recovered.

producing beta-hemolysis were scored Kanagawa phenomenon (KP) positive.

MLSA. The identities of the North Inlet *Vibrionaceae* isolates were determined by multilocus sequence analysis (MLSA) of concatenated 16S rRNA gene, recombinase A (*recA*), RNA polymerase alpha subunit (*rpoA*), and gyrase B (*gyrB*) gene sequences. Bacterial genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI) and diluted to 25 ng μl^{-1}. The PCR program used to amplify *recA*, *rpoA*, and *gyrB* consisted of an initial denaturation at 95°C for 5 min, three cycles of 95°C for 1 min, 55°C for 2 min 15 s, and 72°C for 1 min 15 s, and then 30 cycles of 95°C for 35 s, 55°C for 75 s, and 72°C for 75 s, and a final elongation at 72°C for 7 min. *recA* (~790 bp) and *rpoA* (~900 bp) amplification employed the primers of Thompson et al. (43), and *gyrB* (~800 bp) amplification employed the primers of Ast and Dunlap (44). 16S rRNA (~1,500 bp) was amplified using the primers and protocols of Lane (45). PCR products were resolved on a 1.5% agarose gel and sequenced using an ABI Prism 3730 DNA analyzer. Sequences were edited and neighbor-joining trees were constructed using the Kimura 2-parameter model with Mega version 5.05 (46). Sequence data of reference *Vibrionaceae* species were obtained from the NCBI GenBank.

Recovery and analysis of hemolysin and T3SS2 genes. All PCRs targeting *tdh*, *trh*, and *tlh* were carried out in 25-μl volumes using *Taq* DNA polymerase (Qiagen, Valencia, CA). The PCR thermocycling program, conditions, and primers of Bej et al. (15) were employed for amplification of *tlh*. PCR amplifications of the *tdh* and *trh* genes used the primers of Gutierrez West et al. (26) and were performed in separate reactions. Each reaction mix included 1× PCR buffer (Qiagen), 1.25 U of *Taq*, 0.5 μM each primer, and 200 μM each deoxynucleoside triphosphate (dNTP) (Qiagen) and yielded single bands of 245 bp (*tdh*) or 410 bp (*trh*). The thermal cycling program for the three hemolysin genes was denaturation at 95°C for 5 min, 40 cycles consisting of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final elongation of 72°C for 2 min.

The T3SS2α gene *vscC2* (VPA1339) encodes an outer membrane protein and has been used to determine the presence of T3SS2α, which is correlated with enterotoxicity due to *V. parahaemolyticus* (28, 32). Primers designed by Noriea et al. (32) were used to amplify an approximately 330-bp segment of *vscC2* in 25-μl volumes using *Taq* DNA polymerase (Qiagen, Valencia, CA). Each reaction mixture included 10× PCR buffer (Qiagen), 0.125 U of *Taq*, 1.25 μM primer, 0.5 μM each dNTP (Qiagen), and 2 μM MgCl. The thermal cycling program used for detection of *vscC2* was denaturation at 94°C for 4 min, 32 cycles consisting of 94°C for 45 s, 60°C for 40 s, and 72°C for 45 s, and a final elongation at 72°C for 7 min.

PCR products were sequenced, the resulting gene sequences were edited, and Neighbor-joining trees were constructed using the Kimura 2-parameter model with Mega version 5.05 (46).

Nucleotide sequence accession numbers. Sequence data obtained from this work were submitted to the NCBI GenBank and assigned the following accession numbers: JX453017 to JX453108, JX257004 to JX257016, JX262950 to JX262990, KF197044 to KF197068, KF569811 to KF569825, and KF578086 to KF578118.

RESULTS

Fifty-five strains of vibrios produced green (non-sucrose-fermenting) colonies on TCBS agar. MLSA demonstrated that 18 of these strains were not *V. parahaemolyticus* (Fig. 1). Thirteen of the 18 strains from the North Inlet estuary yielded negative results for β-galactosidase activity and sucrose fermentation and grew at 42°C, as would be expected for *V. parahaemolyticus* (42). The other five strains produced green colonies on TCBS agar, fermented sucrose to acid end products in broth, lacked β-galactosidase activity, and grew at 42°C.

Twelve of the North Inlet strains were identified as *Vibrio diabolicus*; these strains grouped with the *V. diabolicus* type strain HE800^T in the MLSA tree. Three strains grouped with the *Vibrio tubiashii* type strain LMG 10936^T but were not similar enough to the type strain to be considered true *V. tubiashii* strains. These three strains (JPW-8-11-4, JPW-9-11-6, and JPW-9-11-11) most likely represent a new species of *Vibrio* that is related to *V. tubiashii* and are referred to in the present study as *V. tubiashii*-like. The other *Vibrionaceae* recovered from the North Inlet were strains of *Vibrio harveyi* (TBS-9-11-8), *Vibrio natriegens* (TBW-8-11-5), and *Photobacterium damselae* subsp. *damselae* (JPW-8-11-6).

All 18 strains were screened for the three hemolysin genes (Table 1). Six *tlh* amplicons were detected. Two strains produced faint amplicons that, after multiple attempts, did not yield sequence

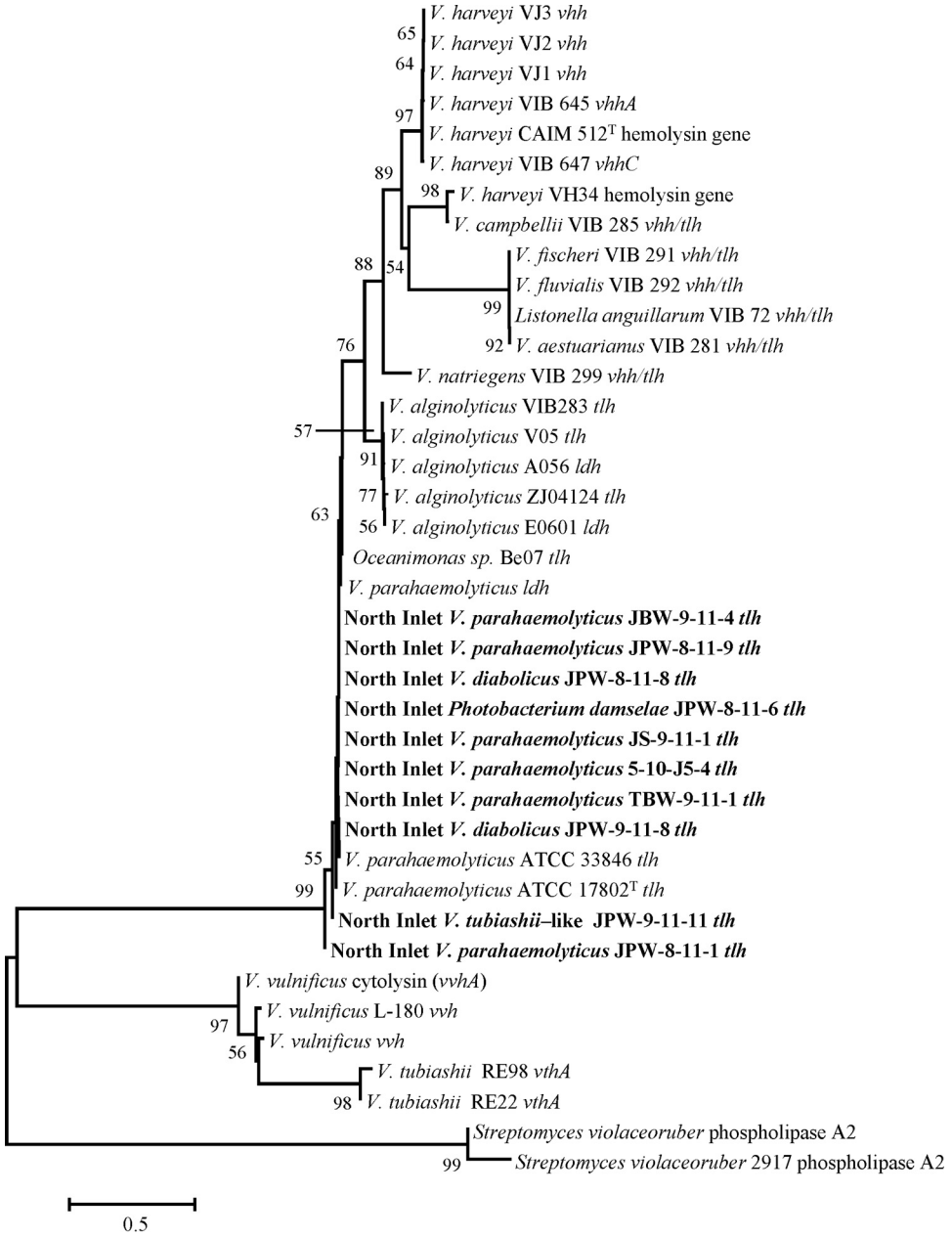

FIG 2 Neighbor-joining tree (Kimura 2-parameter model) of *tlh* gene sequences from environmental *Vibrionaceae* isolates. Gene sequences of hemolysins homologous to *tlh* are also shown. Bootstrap values represent 1,000 replications, and values less than 50 are not shown. Phospholipase A2 gene sequences from *Streptomyces violaceoruber* served as the outgroup. Reference sequences were acquired from the NCBI GenBank.

data. This has been observed previously for some authentic *V. parahaemolyticus* strains (26). *tlh* gene sequences were recovered from the other four strains (Fig. 2). The *V. diabolicus* strains JPW-9-11-8 and JPW-8-11-8 contained *tlh* sequences that were each 99.3% similar to *tlh* gene sequence from the *V. parahaemolyticus* type strain ATCC 17802^T. *tlh* was also detected in JPW-9-11-11 (*V. tubiashii*-like) and JPW-8-11-6 (*P. damselae*). *tlh* gene sequences from these species were 99.3 and 99.1% similar, respectively, to those from *V. parahaemolyticus* ATCC 17802^T *tlh*.

tdh amplicons were detected in 5 *V. diabolicus* strains and 1 *V. tubiashii*-like strain (Fig. 3). Two of the strains produced faint amplicons that after multiple attempts did not yield sequence data, indicating that these strains contain a variant of *tdh*. Four amplicons, all from *V. diabolicus*, yielded sequence data. Three of the *tdh* gene sequences had high similarities (97.6 to 99.2%) to the *tdh* sequence of the *V. parahaemolyticus* reference strain ATCC 33846, which was employed because the *V. parahaemolyticus* type strain ATCC 17802^T does not contain *tdh*. The *tdh* sequence from CW-9-11-1 appeared to be divergent, having only 83.2% similarity to the ATCC 33846 *tdh* sequence. The translated peptide encoded by the CW-9-11-1 *tdh* gene sequence was 75.9% similar to the peptide sequence from *V. parahaemolyticus* 33846. The hemolysin gene *trh* was not recovered from any of the non-*V. parahaemolyticus* strains examined in this study. This gene was also less

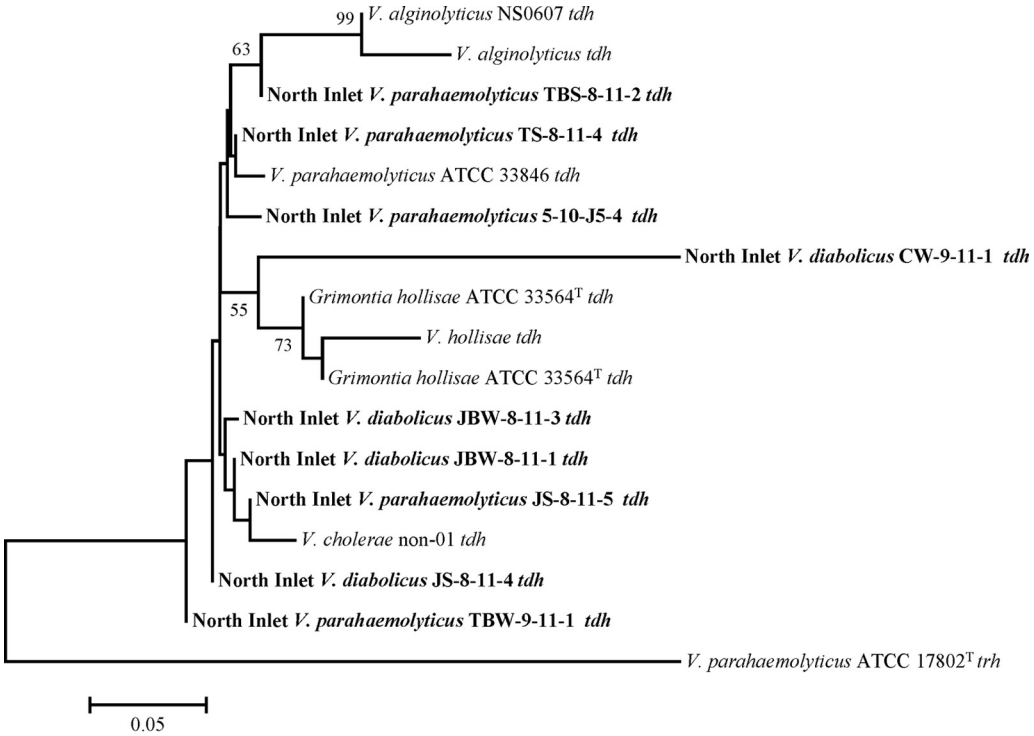

FIG 3 Neighbor-joining tree (Kimura-2 parameter model) of *tdh* gene sequences recovered from environmental *Vibrionaceae* isolates. Bootstrap values represent 1,000 replications, and values less than 50 are not shown. Reference sequences were obtained from the NCBI GenBank; the *trh* gene sequence of ATCC 17802^T served as the outgroup.

common in North Inlet *V. parahaemolyticus* strains than *tlh* or *tdh* (26).

The T3SS2α gene *vscC2* was detected at the highest frequency of the genes examined. Twelve of the 18 non-*V. parahaemolyticus* strains (67%) contained *vscC2*. *vscC2* was detected in 7 *V. diabolicus* strains, 2 *V. tubiashii*-like strains, a *P. damselae* strain, a *V. natriegens* strain, and a *V. harveyi* strain (Fig. 4). All of the *vscC2* amplicons yielded sequences that were highly similar (92.9 to 98.8%) to the *vscC2* sequence from the *V. parahaemolyticus* clinical strain RIMD 2210633, in which *V. parahaemolyticus* T3SS2 was first described (27). In total, at least one *V. parahaemolyticus*-like virulence gene was detected in 16 of the 18 non-*V. parahaemolyticus* strains (89%) from North Inlet. None of the genes of interest were detected in two of the *V. diabolicus* strains (see below). These two additional strains (*V. diabolicus* strains JBS-8-11-1 and TBS-8-11-1) did not contain virulence factor genes but were included because they had virulence-related phenotypic characters (Table 1).

Seven of the 18 non-*V. parahaemolyticus* strains (≥38%) were KP positive. Three of these KP-positive strains contained *tdh*. The four strains that did not produce a *tdh* amplicon but were KP positive were JPW-9-11-6, JBW-9-11-8, TBS-9-11-8, and JBS-8-11-1, and three of these strains contained other virulence-related genes. *vscC2* was detected in JBW-9-11-8 (*V. diabolicus*) and TBS-9-11-8 (*V. harveyi*). *tlh* was detected in JPW-9-11-6 (*V. tubiashii*-like), but none of the four marker genes was detected in JBS-8-11-1 (*V. diabolicus*). Additionally, three strains that contained *tdh* did not produce a KP-positive phenotype.

DISCUSSION

All of the non-*V. parahaemolyticus* environmental *Vibrionaceae* strains examined in this study were identical in appearance to *V. parahaemolyticus* on TCBS agar, lacked β-galactosidase activity, and were able to grow at 42°C. Five strains fermented sucrose to acid end products in broth but were phenotypically identical to *V. parahaemolyticus* for all other tests. TCBS agar is a medium commonly used in the isolation of clinical and environmental vibrios; however, only 67% of the presumptive *V. parahaemolyticus* colonies were confirmed by *recA* phylogenetic analysis (26) to be *V. parahaemolyticus*. Clearly, results of TCBS agar plating and confirming physiological testing of the kind used here must be supported by further analysis to determine the identities of environmental strains. It should be noted that ≥38% of the non-*V. parahaemolyticus* strains examined demonstrated the Kanagawa phenomenon, which is also inadequate for precise identification of this species.

The thermolabile hemolysin gene *tlh* has provided a high-throughput and convenient means to determine *V. parahaemolyticus* numbers in samples, but this analysis is clearly subject to false positives arising from other *Vibrio* species. Unlike previous studies (34), the *tlh* sequence data recovered from non-*V. parahaemolyticus* strains indicate that *tlh* gene sequences in *V. diabolicus*, *V. tubiashii*-like, and *P. damselae* are highly similar to those in *V. parahaemolyticus*. This makes unambiguous species discrimination, even employing gene sequences, much more difficult. The mol% G+C content of *tlh* (47.6%) (13) corresponds to the mol% G+C contents of these four *Vibrionaceae* species (43 to 49%) (42), which indicates that any horizontal transfer of *tlh* within this family occurred far enough in the past for genetic drift to eliminate clear-cut evidence of this process. Some previous reports of *tlh* in non-*V. parahaemolyticus* vibrios attributed its detection to false-positive results (47). False-positive PCR amplifi-

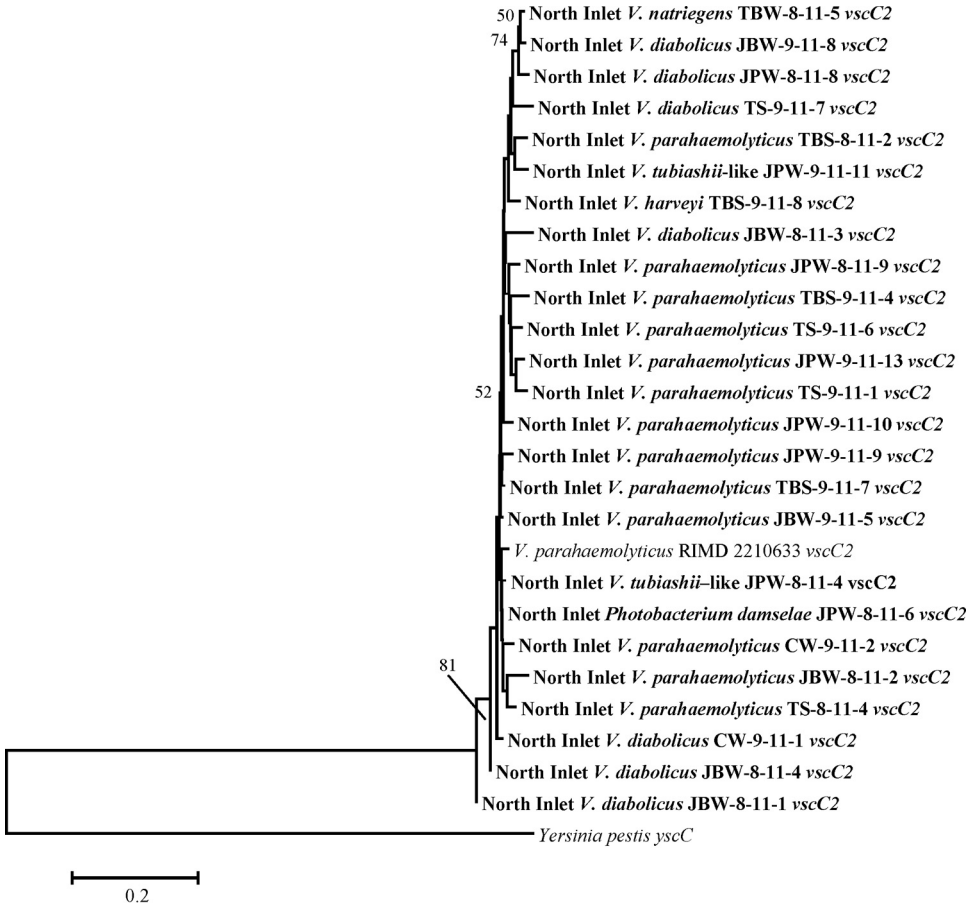

FIG 4 Neighbor-joining tree (Kimura 2-parameter model) of *vscC2* gene sequences recovered from environmental *Vibrionaceae* isolates. Bootstrap values represent 1,000 replications; values less than 50 are not shown. The *yscC2* gene sequence of *Yersinia pestis* served as the outgroup, and reference sequences were obtained from the NCBI GenBank.

cation arising from sample contamination is certainly possible, but true *tlh* amplicons may have been recovered instead. Additionally, *tlh* is highly similar to homologous hemolysin genes found in other *Vibrio* species, including *V. harveyi* (*vhh*), *V. tubiashii* (*vth*), and *V. vulnificus* (*vvh*). Wang et al. (36) determined that DNA probes for *tlh* and *vhh* could be used interchangeably, demonstrating that *tlh* probing for *V. parahaemolyticus* lacks the necessary specificity. Using *tlh* as the sole marker for *V. parahaemolyticus* densities may lead to overestimation of total *V. parahaemolyticus* counts by including other *Vibrio* species carrying *tlh* or similar hemolysins (*V. harveyi*, *V. tubiashii*, and *V. vulnificus*). The extent of this overestimation may be variable among different systems and sample types. It appears that no virulence factor gene sequence can be used to detect *V. parahaemolyticus* quantitatively without additional, supporting analyses.

The thermostable direct hemolysin gene (*tdh*) and a T3SS2α gene, both typical of virulent *V. parahaemolyticus* strains, are also not restricted to this species. In fact, virulence features generally attributed to *V. parahaemolyticus* were common in environmental *Vibrionaceae* strains that were isolated from an estuary having negligible human impacts. The apparent rarity of thermostable hemolysin genes in environmental *V. parahaemolyticus* strains was refuted by Gutierrez-West et al. (26). Results of the present study extend those observations, as frequencies of these genes in environmental non-*V. parahaemolyticus* are also quite high. The *Vibrionaceae* species in which these toxin genes were detected also expand the list of species that carry genes correlated with pathogenicity. *tdh* sequences have been recovered from clinical strains of *Vibrio cholerae* non-O1, *Vibrio mimicus*, and *Vibrio hollisae* (33), but environmental strains also carry this gene. Non-*V. parahaemolyticus tdh* sequences recovered in this study were similar to those of *V. parahaemolyticus tdh*, with the exception of *tdh* from *V. diabolicus* CW-9-11-1, which was clearly divergent from the other sequences and might represent a variant of *tdh*. It seems likely that these genes were transferred from an origin taxon to other *Vibrio* species, including *V. parahaemolyticus* and perhaps others. Phenotypic manifestation of *tdh* has frequently been demonstrated with Wagatsuma agar via the KP, but there was a poor correlation in these strains between *tdh* detection and KP. Three strains contained *tdh* yet were KP negative, and three strains were KP positive yet yielded negative results for canonical hemolysin gene detection. These results indicate that either detection of these virulence-related genes was faulty or other virulence mechanisms are responsible for the KP-positive result from JBS-8-11-1.

Recent studies have established a correlation between T3SS2 and hemolysin genes in *V. parahaemolyticus*, with T3SS2α distribution correlated with the presence of *tdh* in KP-positive stains and T3SS2β with *trh* (31, 32). We found no correlation between

the T3SS2α marker gene *vscC2* and the presence of *tdh*. *vscC2* was detected at high frequency in environmental *Vibrionaceae*. The *vscC2* gene sequences from *V. parahaemolyticus* strains are highly similar to those from non-*V. parahaemolyticus* strains, indicating a recent transfer of this gene. This gene has been reported in other vibrios, including *V. cholerae* non-O1 and *V. mimicus* strains (31, 37). Evidence of *vscC2* in *V. diabolicus*, *P. damselae*, *V. natriegenes*, *V. harveyi*, and *V. tubiashii*-like strains greatly expands the known distribution of the T3SS2α gene *vscC2* in *Vibrionaceae*.

V. parahaemolyticus hemolysin genes and T3SS2 genes have been detected previously in the highly pathogenic *Vibrio cholerae* non-O1 strain but also in vibrios that rarely, if ever, cause illness in humans, including *V. alginolyticus*, *V. mimicus*, and *V. hollisae* (33–35, 37–39, 48). None of the non-*V. parahaemolyticus* strains we isolated belong to a species implicated in human infections, being either pathogenic to marine fauna (*V. tubiashii*, *V. harveyi*, and *P. damselae*) or considered to be nonpathogenic (*V. diabolicus* and *V. natriegens*). The *V. diabolicus* strains are of particular interest, because this species has seldom been reported since its description by Raguenes et al. (49). The frequent recovery of *V. diabolicus* strains from the pristine North Inlet estuary suggests that this species is more broadly distributed than previously thought, and the finding of *V. parahaemolyticus*-like virulence factor genes in this species implicates it as a reservoir for these genes. Additionally, the high similarity of the *V. diabolicus* and *V. parahaemolyticus tdh* and *vscC2* sequences indicates that these genes are readily transferred among these and other *Vibrionaceae* species.

Maintenance of superfluous virulence factor genes in the absence of positive selection is unlikely, indicating other potential roles of these virulence factors in the environment. The pristine North Inlet estuary is a strongly nutrient-limited environment (26, 50). Virulence factors implicated in diseases of humans could also be used to acquire nutrients through damage to cells of estuarine organisms, and several *Vibrio* species may be able to acquire nutrients in this manner. If these species are ingested and expression of these genes and function of the gene products in human hosts occur, the emergence of pathogenic potential in these vibrios due to *V. parahaemolyticus*-related virulence factors would be confirmed. Shellfish-harvesting practices are based on the established seasonal population dynamics of environmental *V. parahaemolyticus*, which grows optimally in the warmer months. Transfer of *V. parahaemolyticus* virulence factors to other vibrios with different optimal growth conditions or geographic distributions potentially changes the dynamics of virulent *Vibrio* populations. Most research on the well-known pathogenic vibrios, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, has focused on clinical and epidemiological impacts of the organisms, but if the addition of virulence factors to the genomes of *Vibrio* species that are not generally considered pathogenic is increasing their fitness in the environment, additional virulent *Vibrio* species, perhaps with different environmental preferences and different host ranges, could emerge.

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